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(54) Title: COMPOSITIONS AND METHODS FOR TREATING OR PREVENTING INFECTIONS IN CANINE AND FELINE ANIMALS

(57) Abstract

Compositions and methods for treating or preventing infections in canine or feline animals which comprises administering an effective amount of granulocyte colony stimulating factor (G-CSF), are disclosed. The G-CSF may be naturally derived, or alternatively, the G-CSF and genetically engineered variance of G-CSF may be the expression products of genetically engineered prokaryotic or eukaryotic host cells.

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Ala Pro Leu Gly Pro Thr Gly Pro Leu Pro Gln Ser Phe Leu Leu
gcc ccc ctg ggc cct acc ggc ccc ctg ccc cag agc ttc ctg ctc

Lys Cys Leu Glu Gln Met Arg Lys Val Gln Ala Asp Gly Thr Ala
aag tgc cta gag caa atg agg aag gtc cag gct gat ggc acg gcg

Leu Gln Glu Thr Leu Cys Ala Thr His Gln Leu Cys His Pro Glu
ctg cag gag acg ctg tgt gcc acc cac cag ctg tgc cat cct gag

Glu Leu Val Leu Leu Gly His Ala Leu Gly Ile Pro Gln Pro Pro
gag ttg gtg ctg ctc ggg cac gct ctg ggc atc ccc cag cct ccc

Leu Ser Ser Cys Ser Ser Gln Ala Leu Gln Leu Met Gly Cys Leu
ctg agc agc tgc tcc agc cag gcc ctg cag ctg atg ggc tgc ctg

Arg Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln
cgt caa ctc cac agc ggc ctc ttc ctc tac cag ggc ctc ctg cag

Ala Leu Ala Gly Ile Ser Pro Glu Leu Ala Pro Thr Leu Asp Thr
gcc ctg gca ggg ata tcc ccc gag tta gcg ccc acc ttg gac aca

Leu Gln Leu Asp Thr Thr Asp Phe Ala Ile Asn Ile Trp Gln Gln
ctg cag ctg gac acc acc gac ttt gcc atc aac atc tgg cag cag

Met Glu Asp Leu Gly Met Ala Pro Ala Val Pro Pro Thr Gln Gly
atg gaa gat cta gga atg gcc ccc gcc gtg cca cct acc cag ggc

Thr Met Pro Ala Phe Thr Ser Ala Phe Gln Arg Arg Ala Gly Gly
acc atg cca gcc ttc acc tcg gcc ttc cag cgc cgg gca gga ggt

Val Leu Val Ala Ser Asn Leu Gln Ser Phe Leu Glu Leu Ala Tyr
gtc ctg gtg gcc tcc aac ctg cag agc ttc ctg gag ctg gca tat

Arg Ala Leu Arg His Phe Ala Lys Pro
cgc gct ctg cgc cac ttt gcc aaa ccc
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COMPOSITIONS AND METHODS FOR TREATING OR PREVENTING INFECTIONS IN CANINE AND FELINE ANIMALS

Field Of The Invention

The present invention is directed to the use of granulocyte colony stimulating factor (G-CSF) to treat or prevent infections in canine and feline animals. More specifically, the invention is directed to the use of G-CSF having the amino acid sequence of human G-CSF or having the amino acid sequence of canine G-CSF, in treating or preventing infections in canine or feline animals. The source of the G-CSF may be naturally derived or may be derived from genetically engineered prokaryotic or eukaryotic host cells containing recombinant plasmid or viral DNA vectors carrying the human or canine G-CSF gene, or genetically engineered variants of canine G-CSF genes, or synthetic human or canine G-CSF genes. The present invention is also directed to DNA gene segments, biologically functional recombinant plasmids and viral DNA vectors, and prokaryotic and eukaryotic host cells containing such recombinant plasmids and vectors, all of which contain a canine G-CSF gene or a genetically engineered variant of a canine G-CSF gene.

Background Of The Invention

Although antibiotic therapy is now used for animal infections with some success, huge losses persist. The early hopes that antibiotics would allow complete control of the disease have not been realized. None of the antibiotics utilized thus far has been entirely satisfactory. Additionally, it has been found to be very desirable to replace antibiotic treatment with treatment by non-antibiotic chemo-therapeutic drug compounds, for the following reasons:

(1) Antibiotics effective in human medicine should not be utilized in veterinary medicine, in order not to build up strain resistance of bacteria appearing in human diseases; and

(2) Antibiotics should be reserved for such diseases for which no chemo-therapeutic drug compound would be available, as it has been proved that bacterial strains build up resistance to an antibiotic after extended use of such antibiotic.

Despite these several published methods, it remains very important to find cost-effective methods utilizing non-antibiotic compounds which would substantially overcome the drawbacks of antibiotics used thus far and yet would be effective in treating and preventing infections in canine and feline animals.

Canine parvo virus still infects over one-half million young dogs. Hospitalization and intensive care are required. Mortality occurs in 15-20% of the cases. Severe neutropenia occurs and death is thought to frequently result from secondary infections and sepsis.

Feline Immunedeficiency Virus (FIV) is believed to infect 500,000-1,000,000 cats per year. This virus causes neutropenia in approximately 30% of the cats which renders them susceptible to infections. Feline Leukemia Virus (FeLV) also causes neutropenia in cats.

Granulocyte Colony Stimulating Factor

Granulocyte colony stimulating factor (G-CSF) is one of several glycoprotein growth factors known as colony stimulating factors (CSFs) because they support th prolif ration of haemopoietic progenitor cells. G-CSF stimulates the proliferation of specific bone marrow precursor cells and their differentiation into granulocytes. It is distinguished from other CSFs by

its ability to both stimulate neutrophilic granulocyte colony formation in semi-solid agar and to induce terminal differentiation of murine myelomonocytic leukemic cells in vitro. The cDNA cloning and expression of recombinant human G-CSF has been described, and it has been confirmed that the recombinant G-CSF exhibits most, if not all, of the biological properties of the native molecule (Souza, L. et al. Science 232, 61-65 (1986)). Sequence analysis of the cDNA and genomic DNA clones has allowed the deduction of the amino acid sequence and reveals that the protein is 204 amino acids long with a signal sequence of 30 amino acids. The mature protein is 174 amino acids long and possesses no potential N-linked glycosylation sites but several possible sites for O-linked glycosylation.

The cloning and expression of cDNA encoding human G-CSF has been described by two groups (Nagata, S. et. al., Nature 319, 415-418 (1986); Souza, L. M. et al., Science 232, 61-65 (1986)). The first report of a G-CSF cDNA clone suggested that the mature protein was 177 amino acids in length. The authors reported that they had also identified a cDNA clone for G-CSF that coded for a protein that lacked a stretch of three amino acids. This shorter form of G-CSF cDNA expresses the expected G-CSF activity. The second report describes a cDNA sequence identical to this short form and makes no mention of other variants. Since these authors confirmed that the short cDNA expresses G-CSF with the expected profile of biological activity, it is probable that this is the important form of G-CSF and that the longer form is either a minor splicing variant or the result of a cloning artifact.

Matsumoto et al., in Infection and Immunity, Vol. 55, No. 11, p. 2715 (1987) discuss the protective effect of human G-CSF on microbial infection in neutropenic mice.

The following patent publications relate to G-CSF: WO-A-8703689, assigned to Kirin/Amgen describes hybridomas producing monoclonal antibodies specific for G-CSF and their use in the purification of G-CSF; WO-A-8702060, assigned to Biogen, discloses human G-CSF like polypeptides and methods of producing them; U.S. Patent 4,810,643 assigned to Amgen, discloses human G-CSF like polypeptides, sequences encoding them and methods of their production; and WO-A-8604605 and WO-A-8604506, both asigned to Chugai Seiyaku Kabushiki Kaisha, disclose a gene encoding human G-CSF and infection inhibitors containing human G-CSF.

The use of recombinant G-CSF with the same amino acid sequence as human G-CSF, in dogs with cyclic neutropenia has been associated with the development of neutralizing antibodies to the heterologous G-CSF protein during a thirty day period of administration (see Lothrop et al., Blood 72, 5624-37 (1988). Subsequent treatment of these same dogs with recombinant human GM-CSF failed to cause a significant leukocytosis or eliminate cycles of neutropenia. A significant variation in structure may explain the development of neutralizing antibodies when the human sequence products are given to dogs. The development of neutralizing antibodies in dogs given the human sequence products may limit them to single or short term use.

It is an object of the subject invention to provide an improved method of treating and preventing infections in canine or feline animals.

It is a further object of the subject invention to provide a method of treating infections in canine or feline animals without build up of strain resistance of bacteria.

A still further object of the invention is to provid a purified and isolated polypeptide having part or all of the primary structural conformation and the

biological properties of naturally occurring canine G-CSF, and DNA sequences encoding such G-CSF.

Other objects, features and characteristics of the present invention will become apparent upon consideration of the following description and the appended claims.

Summary Of The Invention

The present invention provides DNA sequences, biologically functional recombinant plasmids and viral DNA vectors, and prokaryotic and eukaryotic host cells containing such recombinant plasmids and vectors, all of which contain a canine G-CSF gene or a genetically engineered variant of a canine G-CSF gene. The invention also provides polypeptides encoded by the canine G-CSF gene or variants thereof. A method for treating or preventing infections in canine or feline animals is also disclosed.

Novel DNA sequences of the invention include sequences useful in securing expression in prokaryotic or eukaryotic host cells of polypeptide products having at least a part of the primary structural conformation and the biological properties of naturally occurring canine granulocyte colony stimulating factor. DNA sequences of the invention are specifically seen to comprise the DNA sequence of the coding region of the mature protein, set forth in Figure 2 or its complimentary strand, allelic variant forms of canine G-CSF, manufactured DNA sequences encoding canine G-CSF, fragments of canine G-CSF and analogs of canine G-CSF with DNA sequences incorporating codons facilitating translation of messenger RNA in microbial hosts. Such manufactured sequences may readily be constructed according to the methods of Alton, et al., PCT published application WO 83/04053.

A further embodiment of the invention relates to synthetic genes designed to allow for expression of G-CSF having the canine amino acid sequence in E. coli.

Also comprehended by the invention are pharmaceutical compositions comprising effective amounts of polypeptide products of the invention together with suitable diluents, adjuvants and/or carriers useful in animal therapy.

The subject invention also relates to a method for treating and preventing infections in canine or feline animals by administering a therapeutically effective treating or preventing amount of granulocyte colony stimulating factor, advantageously G-CSF derived from the gene of a canine animal. In addition, the invention relates to a method of treating cancer in canine or feline animals by administering a therapeutically effective treating or preventing amount of granulocyte colony stimulating factor as an adjunct to chemotherapy.

Brief Description of the Drawings

Figure 1 shows the restriction map of canine G-CSF;

Figure 2 illustrates the coding region of the mature protein of canine G-CSF;

Figure 3 is the genomic sequence of the human G-CSF;

Figure 4 is the DNA sequence of a canine G-CSF synthetic gene (cG-CSF dna);

Figure 5 illustrates the oligos used to construct the subunits of the canine G-CSF synthetic gene (cG-CSF dna3);

Figures 6A and 6B shows the two subunits of the canine G-CSF synth tic gene cG-CSF dna3;

Figure 7 shows the homology of canine and human G-CSF.

Figures 8-9 are graphic representations of the results obtained in Example 5 which relates to treatment of dogs with G-CSF having the canine amino acid sequence.

Detailed Description Of The Invention

A novel method for treating or preventing infections in canine or feline animals has been discovered. Surprisingly it has been found that G-CSF is effective in a method of treating or preventing infections in canine and feline animals.

The subject invention also relates to treating cancer in dogs or cats by administration of G-CSF as an adjunct to chemotherapy, advantageously, as an adjunct to the use of myelosuppressive drugs. The general method as it applies to humans is described in Gabrilove et al., New England Journal of Medicine 318, No. 22 (1988) hereby incorporated by reference. A skilled veterinarian will adjust the method of administrating dose etc. as appropriate.

A variety of infections afflicting canine and feline animals are treatable by the method of the subject invention. A veterinarian of ordinary skill can readily determine whether an animal exhibits an infection. In one embodiment, the present invention relates to a method of treating or preventing infections such as Feline Immunodeficiency Virus (FIV) in feline animals comprising administering a composition which comprises an effective amount of G-CSF.

In another embodiment of the invention, G-CSF is used to treat Feline Leukemia Virus (FeLV). Additionally G-CSF is used to treat cats with Pan Leukopenia.

In another embodiment dogs infected with Parvo Virus are treated with G-CSF.

The subject invention also relates to the use of G-CSF during bone marrow transplants. G-CSF shortens the time to engraftment (4-7 days vs. 7-10 days in a study with 12 cats).

By "G-CSF" is meant one of the hematopoietic growth factors known as granulocyte colony stimulating factors. The biological activities of G-CSFs include: stimulating the differentiation of a small number of progenitor "stem cells" into the variety of blood cell lines, stimulating the proliferation of those blood cell lines and stimulating the ultimate differentiation of mature blood cells from those lines. The preferred G-CSF polypeptides for treating or preventing infections in canine or feline animals are human and canine, and may be naturally-derived or the product of genetically engineered host cells containing a DNA sequence encoding G-CSF.

The DNA encoding the G-CSF gene is a genomic DNA sequence, a cDNA sequence or a manufactured (or synthetic) DNA sequence which is expressed in a prokaryotic or eukaryotic host cell as a polypeptide having part or all of the primary structural conformation and the hematopoietic biological properties of naturally-occurring G-CSF. A biologically functional plasmid or viral DNA vector containing a DNA sequence encoding G-CSF may be used to transform or transfect a prokaryotic or eukaryotic host cell to produce cell lines expressing the G-CSF polypeptide, glycosylated or unglycosylated.

The various forms of G-CSF, including their preparation and purification, useful in a method for treating or preventing infections in canine or felin animals commonly owned are described in detail in U.S. Patent 4,810,643, which is hereby incorporated by

reference. U.S. 4,810,643 describes and claims novel gene segments, biologically functional recombinant plasmids and viral DNA vectors and prokaryotic and eukaryotic host cells, which contain a G-CSF gene or a genetically engineered variant of a G-CSF gene. The host cells express biologically active G-CSF or a genetically engineered variant of G-CSF.

This application describes the isolation and characterization of a canine G-CSF gene and in particular describes and claims novel gene segments, biologically functional recombinant plasmids and viral DNA vectors, and prokaryotic and eukaryotic host cells, which contain a canine G-CSF gene or a genetically engineered variant of a canine G-CSF gene. The host cells transformed or transfected with the recombinant plasmids or viral DNA vectors express biologically active G-CSF or a genetically engineered variant of G-CSF. The protein expressed is purified using methods known to those skilled in the art.

DNA sequences coding for all or a part of G-CSF having the canine amino acid sequence are provided. Such DNA sequences include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts (e.g. E. coli preferred codons, see Nucleic Acids Res. 1986 vol.14 (13) pp 5125-5143); the provision of sites for cleavage by restriction endonuclease enzymes; the provision of DNA sequences which reduce or eliminate secondary structure interactions which inhibit transcription and/or translation; and the provision of additional initial, terminal or intermediate DNA sequences which facilitate incorporation into expression vectors. The DNA sequences of the invention also include sequences having an optimized ribosome binding site, and sequences which enhance transcription, translation, and/or secretion of the protein product.

The present invention also provides DNA sequences coding for expression of polypeptide analogs or derivatives of canine G-CSF which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (i.e., deletion analogs containing less than all of the residues specified for canine G-CSF; substitution analogs, wherein one or more residues specified are replaced by other residues; and in addition, analogs wherein one or more amino acid residues are added to a terminal or medial portion of the polypeptide) and which share the properties of naturally-occurring forms.

Also comprehended by the present invention is that class of polypeptide coded for by portions of the DNA complement to the top strand canine cDNA of Figure 2, i.e., "complementary inverted proteins" as described by Tramontano, et al., Nucleic Acids Res., 12, 5049-5059 (1984).

The present invention relates to purified and isolated polypeptide products having part or all of the primary structural conformation (i.e., continuous sequence of amino acid residues) and the biological properties (e.g., immunological properties and in vitro biological activity) of naturally-occurring canine G-CSF including allelic variants thereof. These polypeptides are also characterized by being the product of chemical synthetic procedures or of procaryotic or eukaryotic host expression (e.g., by bacterial, yeast, higher plant, insect and mammalian cells (e.g. CHO or COS) in culture) of exogenous DNA sequences obtained by genomic or cDNA cloning or by gene synthesis. The products of typical yeast (e.g., Saccaromyces cerevisiae) or prokaryote [e.g., [Escherichia coli (E. coli)]] host cells are free of association with any mammalian proteins. Depending upon the host employ d, polypeptide of the invention is glycosylated with mammalian or other

eukaryotic carbohydrates or is non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue (at position -1).

In addition to the recombinant versions of naturally-occurring allelic forms of canine G-CSF, the present invention also embraces other G-CSF products such as polypeptide analogs of canine G-CSF and fragments of canine G-CSF. All such forms of canine G-CSF may be useful in the method for treating or preventing infections in canine or feline animals. Following the procedures of the published application by Alton, et al. (WO/83/04053), hereby incorporated by reference, one can readily design and manufacture genes coding for microbial expression of polypeptides having primary conformations which differ from that herein specified for, in terms of the identity or location of one or more residues (e.g., substitutions, terminal and intermediate additions and deletions). Alternately, modifications of genomic and cDNA genes are readily accomplished by well-known site-directed mutagenesis techniques which generate analogs and derivatives of canine G-CSF. Such products share the hematopoietic biological properties of canine G-CSF. As examples, products of the invention include those which are foreshortened (e.g., by deletions); or those which are more stable to hydrolysis (and, therefore, have more pronounced or longer lasting effects than naturally-occurring); or which have been altered to delete one (or more) potential site(s) for n-linked or o-linked glycosylation (which result in higher activities for yeast-produced products); or which have one or more cysteine residues deleted or replaced (for example, by alanine or serine residues) and are more easily isolated in active form from microbial systems; or which have one or more tyrosine residues replaced by phenylalanine and bind more or less readily to G-CSF receptors on target

cells. Also comprehended are polypeptide fragments duplicating only part of the continuous amino acid sequence or secondary conformations of canine G-CSF.

According to another aspect of the present invention, the DNA sequence described herein which encodes G-CSF polypeptides is valuable for the information which it provides concerning the amino acid sequence of this canine protein (and similar mammalian proteins) which has heretofore been unavailable. The DNA sequences are also valuable as products useful in effecting the large scale microbial synthesis of G-CSF having the same amino acid sequence as canine G-CSF, by a variety of recombinant techniques. Put another way, DNA sequences provided by the invention are useful in generating new and useful viral and plasmid DNA vectors, new and useful transformed and transfected prokaryotic and eukaryotic host cells (including bacterial, yeast, and mammalian cells grown in culture), and new and useful methods for cultured growth of such microbial host cells capable of expression of G-CSF having the canine amino acid sequence, variants or analogs. DNA sequences of the invention are also suitable materials for use as labelled probes in isolating canine G-CSF and related protein encoding cDNA and genomic DNA sequences of other mammalian species. DNA sequences are also useful in various alternative methods of protein synthesis (e.g., in insect cells) or in genetic therapy in mammals. DNA sequences of the invention are useful in developing transgenic mammalian species which may serve as eukaryotic "hosts" for production of G-CSF and G-CSF products in quantity. (See generally Palmiter, et al., Science, 22(4625), 809-814 (1983)).

Of applicability to canine G-CSF fragments and polypeptide analogs of the invention are reports of the immunological activity of synthetic peptides which substantially duplicate the amino acid sequence extant

in naturally-occurring proteins, glycoproteins and nucleoproteins. More specifically, relatively low molecular weight polypeptides have been shown to participate in immune reactions which are similar in duration and extent to the immune reactions of physiologically significant proteins such as viral antigens, polypeptide hormones, and the like. Included among the immune reactions of such polypeptides is the provocation of the formation of specific antibodies in immunologically active animals. (See, e.g., Lerner, et al., Cell, 23: 309-310 (1981); Ross, et al., Nature, 294: 654-656 (1981); Walter, et al., Proc. Natl. Acad. Sci. (USA), 77: 5197-5200 (1980); Lerner, et al., Proc. Natl. Acad. Sci. (USA), 78: 4882-4886 (1981); Wong, et al., Proc. Natl. Acad. Sci. (USA), 78: 7412-7416 (1981); Green, et al., Cell, 28: 477-587 (1982); Nigg, et al., Proc. Natl. Acad. Sci. (USA), 79: 5322-5326 (1982); Baron, et al, Cell, 28: 395-404 (1982); Dreesman, et al., Nature, 295: 183-190 (1982); and Lerner, Scientific American, 248 (2): 66-74 (1983)). See, also, Kaiser, et al. Science, 223: 249-255 (1984) relating to biological and immunological activities of synthetic peptides which approximately share secondary structures of peptide hormones but may not share their primary structural conformation.

All of the above mentioned forms, fragments, variants and analogs of canine G-CSF may be useful in the method of treating or preventing infections in canine or feline animals as described herein.

In another embodiment of the invention, one or more additional colony stimulating factors are administered to the infected animal with G-CSF, egs. GM-CSF, M-CSF, multi-CSF (IL-3). The CSFs are administered together or separately. In a further embodiment, animal infections are treated by administering G-CSF with one or mor of: the interf rons

(advantageously α-interferon), IL-2, IL-6 and TNF or with a traditional antibiotic.

This application also describes pharmaceutical compositions of G-CSF having the canine amino acid sequence in a pharmaceutically acceptable carrier. These compositions may be administered intravascularly, intraperitoneally, subcutaneously, intramuscularly, or by infusion using forms known to the pharmaceutical art. For intravascular, intraperitoneal, subcutaneous, or intramuscular administration, active drug components may be combined with a suitable carrier such as water, saline, aqueous dextrose, and the like. Regardless of the route of administration selected, the compositions of the present invention are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those skilled in the art. An advantageous formulation is disclosed in commonly owned Ser. No. 285,159, hereby incorporated by reference. In one embodiment, sustained release formulations are used.

In one embodiment of the invention, G-CSF treatment is used in a prophylactic manner. For example, dogs or cats are treated prior to occurrences which may debilitate them, in order to boost and prime their capacity to fight off infections. Administration of the G-CSF can be made at the time the dogs or cats undergo surgery or radiation, etc.

Several variables will be taken into account by the ordinary artisan in determining the concentration of G-CSF in the therapeutic formulations and dosages to be administered. Variables include administration route and condition of the animal.

The following examples are presented by way of illustration of the invention and are specifically directed to procedures carried out prior to identification of canine G-CSF genomic and cDNA clones, to procedur s resulting in such identification, and to

the sequencing, development of expression systems based on genomic, cDNA and manufactured (or synthetic) genes and verification of expression of G-CSF having the canine amino acid sequence, and analog products in such systems. The method of isolating the canine G-CSF gene described below can also be used to isolate other animal G-CSF genes, which in turn can be used in producing other animal G-CSFs. In addition, the examples illustrate methods for treating or preventing infections in canine animals, comprising administering an effective amount of G-CSF.

EXAMPLE 1

Screening a Genomic Library for the canine G-CSF Gene

In this example, oligonucleotide probes were used to screen for a genomic clone containing a canine G-CSF gene. A phage (EMBL-3) canine genomic library was obtained from Clontech, plated out on E. coli strain NM538, and screened using ^{32}P phosphorylated oligonucleotide probes of the following sequences:

1. TCC CTG CCC CAG AGC TTC CTG CTC AAG TGC TTA GAG CAA GTG AGG AAG ATC CAG, and

2. GCC ATG CCG GCC TTC ACT TCT GCC TTC CAG CGC CGG GCA GGA GGG GTC CTG

A total of approximately 1.0×10^6 phage were plated on 8 22 cm square petri dishes and plaque lifted in duplicate onto Gene Screen Plus transfer hybridization membranes. One set of membranes was hybridized to probe 1 and the other set was hybridizated to probe 2 using the procedures described in Maniatis et al., Molecular Cloning, A Laboratory Manual (Cold Spring

Harbor Laboratory, New York, 1982). Hybridizations were done at 55°C overnight in 6XSSC, 5X Denhardts, 50 µg/ml sheared herring sperm DNA. A total of 1 positive clone was observed which hybridized to both probes. This clone was rescreened until an isolated plaque was obtained and was grown in a 3 liter culture and phage DNA was prepared as described in Maniatus, supra. This DNA was mapped by restriction enzyme digestion and Southern blotting using the radiolabeled probes. The mapping results showed that a Asp718 fragment of about 3700 bases contained the entire G-CSF region. DNA was digested with Asp718 to release an approximately 3700 bp canine G-CSF containing fragment which was subsequently subcloned into pUC19 at the Asp 718 site and further mapped by restriction endonuclease digests and Southern blotting.

A restriction endonuclease map (approximately 3.7 kb) of genomic DNA containing the canine G-CSF gene is shown in Figure 1. The sequence for the entire coding region of the mature canine G-CSF was determined by subcloning fragments into M13 and sequencing them by the dideoxy method described in Sanger et al., Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467 (1977). Sequences were confirmed or extended by utilizing internal primers off of the same clones. The sequence for the coding region was deduced by direct comparison with the human genomic G-CSF sequence (Figure 3) and is shown in Figure 2. Splice juncture sites and amino terminal processing of the protein were assumed to occur at the same places as the human G-CSF. The DNA sequence codes for a mature protein of the same length as the human G-CSF (174 amino acids) and the proteins are 81% homologous (see Figure 7).

EXAMPLE 2

Construction of Synthetic Canine G-CSF Genes and Expression of Such G-CSF Genes

This example relates to preparation of manufactured genes encoding canine G-CSF and including E. coli preference codons, and to expression of such G-CSF.

Synthetic genes were designed to allow for the expression of canine granulocyte colony stimulating factor in E. coli [cG-CSF dna3 (Figures 4-6)]. Canine G-CSF is 174 amino acids in length and is 81% homologus to the human form of G-CSF (174 a.a.).

The gene cG-CSF dna3 (Figures 4-6) was designed with maximum bias for E. coli codon preference. For gene cG-CSF dna3, in addition to the coding sequence, an initiation ATG, leader and terminator sequences and 5' Xbal and 3' BamHl restriction sites were entered. The gene, cG-CSF dna3, was also designed to have minimum secondary interactions and sufficient unique restriction sites for subunit assembly and gene manipulation. BamHl and Pstl sites were incorporated at positions identical to those found in the human G-CSF gene noted in commonly owned U.S. Patent 4,810,643. This allows for generation of unique human/canine hybrid genes and their protein products.

The gene was designed as two subunits (Subunit I (Xbal-HindIII), and Subunit II (HindIII-BamHl) for cloning into sequencing/expression vectors (Figure 6). Subunit I contains a short leader sequence with an Xbal cloning end and the ribosome binding site (RBS). Subunit II contains a pair of redundant stop codons and the BamHl cloning end.

Briefly stated, the protocol employed was generally as set out in the disclosure of co-owned

Alton, et al., PCT Publication No. WO83/04053, which is incorporated by reference herein. The gene was designed for initial assembly of component oligonucleotides into multiple duplexes which, in turn, were assembled into two discrete sections (Figure 6). These sections were designed for ready amplification and, upon removal from the amplification system, could be assembled sequentially or through a multiple fragment ligation into a suitable expression vector.

The construction of Sections I and II is illustrated in Figures 5 and 6. In the construction of Section I, as shown in Figures 5 and 6, 16 oligonucleotides were assembled into 8 duplexes. The 8 duplexes were then ligated to form Section I. It may also be noted in Figure 6 that Section I includes an upstream Xbal sticky end and a downstream HindIII sticky end useful for ligation to amplification and expression vectors and for ligation to Section II.

Section II was constructed as shown in Figures 5 and 6. For this construction, 16 oligonucleotides were assembled into 8 duplexes. The 8 duplexes were then ligated to form Section II as depicted in Figure 6. As also shown in Figure 6, Section II includes an upstream HindIII sticky end and a downstream BamHl sticky end useful for ligating into amplification and expression vectors, and to Section I.

Although any suitable vector may be employed to express this DNA, the expression plasmid pCFM536 may readily be used. This plasmid is described in U.S. Patent No. 4,710,473 hereby incorporated by reference. Control of expression in the pCFM536 plasmid is by means of a lambda pL promoter, which itself may be under the control of a CI857 repressor gene (such as is provided in E. coli strain FM5 (ATCC deposit 53911).

Section I was initially cloned into M13 from Xbal to HindIII and sequenced by the dideoxy method

(Sanger supra). Section II was cloned into M13 from HindIII to EcoR1 and was also sequenced by the dideoxy method. Section I was cut out of M13 from Xba1 to HindIII and Section II was cut out of M13 from HindIII to EcoR1. These two fragments were then ligated with pCFM536 cut from Xba1 to BamH1 and transformed into E. coli strain FM5 to generate pCFM536cG-CSF.

This plasmid contains the λpL promoter/operator region and has a temperature sensitive replicon. When E. coli strain FM5 harboring pCFM536cG-CSF is cultured at 28°C, the plasmid copy number is maintained at 10-20 copies/cell, and transcription from the λpL promoter is regulated by a temperature sensitive repressor. Growth at 42°C results in an increased copy number and release of repression at the λpL promoter. Recombinant G-CSF having the canine sequence begins to accumulate at elevated temperatures as the result of promoter activation and plasmid amplification. The λpL promoter lies just upstream from the ribosome binding site and the methionine initiation codon of canine G-CSF. The transcription terminator, t-oop, lies just downstream from the two translational stop codons near the 3' end of the gene. Strain FM5 harboring the plasmid, pCFM536cG-CSF, expresses recombinant G-CSF having the canine sequence at up to 30% of the total cellular protein.

EXAMPLE 3

Construction of Canine G-CSF Analogs

This example relates to the use of recombinant methods to generate an analog of canine G-CSF wherein the cysteine at position 17 was individually replaced by a serine.

Site directed mutagenesis procedures according to Souza, et al., published PCT Application No. WO85/00817, published February 28, 1985, hereby incorporated by reference, were carried out using the oligonucleotide CTG CTG AAA TCC CTC GAG CAG.

EXAMPLE 4

E. coli Canine G-CSF Purification

The general purification method is disclosed in commonly owned Ser. No. 348,011 hereby incorporated by reference.

Cell Breakage and Sarkosyl Solubilization and Oxidation

About 200 grams of cell paste were weighed out in 1.5 liters of cold water. The cell paste was dispersed with a homogenizer until completely dispersed. The homogenate was then passed through a Gaulin Homogenizer four times at 8000 psig. The material was then centrifuged in the Beckman J2 21 centrifuge using the JA 10 rotor at 9000 rpm for 30 minutes at 4°C. The supernatant was decanted and discarded. The pellet was resuspended in 1.5 liters of cold water and again centrifuged in the Beckman J2 21 centrifuge using the JA 10 rotor at 9000 rpm for 30 minutes at 4°C. The supernatant was decanted and discarded. The pellet was resuspended in 760 mL water and 40 mL 1M Tris, pH 8.0 was added followed by 200 mL 10% Sarkosyl. After this material stirred at room temperature for about ten minutes, 1 mL 1% copper sulfate pentahydrate was added. This material was stirred at room temperature overnight (approximately 16 hours). The material was then centrifuged in the Beckman J2 21 centrifuge using the JA 10 rotor at 9000

rpm for 30 minutes at 4°C. The supernatant was decanted and saved. The pellets were discarded.

Dowex Removal of Sarkosyl

To the supernatant was added 1 liter of cold water and then 2 liters cold 20 mM Tris, pH 8.0 and then 800 grams prepared Dowex (see Ser. No. 348,011 hereby incorporated by reference) was added. This slurry was stirred at 4°C for 90 minutes. The slurry was poured through a column and the flow through collected. The resin was washed with 800 mL cold 20 mM Tris, pH 8.0 which was added to the flow through giving 4800 mL.

DE52 Cellulose Ion Exchange Chromatography

About 4800 mL of material was loaded directly onto a 200 mL DE52 cellulose ion exchange column equilibrated in 20 mM Tris, pH 8.0. The product was eluted off of the column using 100 mM NaCl in 20 mM Tris, pH 9.0. About 1270 mL was collected at approximately 0.8 mg/mL, giving approximately 1 gram.

CM-Sepharose Fast Flow Chromatography

The DE52 100 mM NaCl material was concentrated using a Pellicon system (with a 10,000 MW membrane) to approximately 200 mL. The material was adjusted to pH 5.4 using 50% acetic acid. Six volumes of cold water were added and the material was then loaded directly onto a 50 mL CM-Sepharose Fast Flow ion exchange column equilibrated in 20 mM sodium acetate, pH 5.4. The product was eluted off of the column using a 1 liter gradient from 0-0.2 M NaCl in 20 mM sodium acetate, pH 5.4. About 100 10 mL fractions were collected. Based on the chromatogram results the fractions of

interest were analyzed on a 15% SDS gel. Based on the gel results, fractions 30-51 were pooled giving 258 mL at approximately 2.6 mg/mL, or 685 mgs.

Diafiltration

The CM pool was adjusted to pH 3.5 using 0.1 N HCL and then diafiltered using a Pellicon with a 10,000 MW membrane vs. 0.35 mM HCl-Water. The final volume was adjusted to 685 mL to give material at a final concentration of 1 mg/mL.

EXAMPLE 5

In vivo Activity of Canine G-CSF

Two young adult, healthy mixed breed dogs (one 25 kg male, one 28.6 kg female) were used for this study. The dogs were acclimated to the hospital environment for one week prior to the onset of the study. Complete blood and platelet counts were done three days prior and then immediately prior to the first injection of recombinant cG-CSF. Recombinant E. coli G-CSF having the amino acid sequence of canine G-CSF was diluted in sterile water to 100ug/ml and placed in multiple dose vials. The G-CSF was maintained at 4°C.

A dosage of 5 ug/kg/day was administered subcutaneously to each dog for 4 weeks at the same time each day. Blood for a CBC and platelet count was drawn immediately prior to each G-CSF injection and submitted to the clinical pathology laboratory for evaluation. Daily blood counts were performed until three consecutive daily counts remained stable. Blood was then drawn every other day for two weeks, then ev ry third day th final week.

After 28 days, G-CSF administration was discontinued. Blood counts were followed every other day to determine how rapidly they returned to normal. Once within normal range, G-CSF was started again at the same dosage and administered for another five days to determine the leukocyte response.

Physical examinations were performed on a daily basis. Karnofsky's performance scores were assigned daily to both animals. Body weights and body temperatures were recorded daily. In addition, toxicity evaluation was performed daily. The mean white blood cell count prior to administration of G-CSF was 8,650/ul (neutrophils: 4,880/ul; lymphocytes: 2,398/ul; monocytes: 667/ul; eosinophils: 704/ul; and platelets: 297,000/ul). Twenty-four hours following the first injection of G-CSF, the mean white blood cell count was 39,150/ul (neutrophils: 31,257/ul; neutrophilic bands: 391/ul; lymphocytes: 2,803/ul; monocytes: 2,951/ul; eosinophils: 1,747/ul; platelets: 322,500/ul). This represents a 4.5 fold increase in total white blood cell count within 24 hours. Neutrophils increased by a factor of 6.4 (see Figure 8). Monocytes rose by a factor of 4.4 (see Figure 9). Although the dosage of G-CSF remained at 5 ug/kg/day, an additional increase in blood counts was noted on day eleven. Mean white blood cell count on day nine was 32,550/ul (mean neutrophil count 26,682/ul). On day eleven, the mean white blood cell count was 69,200/ul (mean neutrophil count: 58,764/ul) representing an additional two-fold increase from day nine to day eleven and an eight-fold increase from day one (prior to G-CSF administration). Blood counts remained elevated throughout the 28 day period of administration of G-CSF in one dog. In the second dog ther were 3 days on which decreases in the leukocyte counts wer evident 24 hours aft r administration of a reduced dosage. Counts returned to their pretreatment

levels by the fifth day after G-CSF was stopped. Upon resumption of G-CSF administration, the mean white blood cell count increased by a factor of 6.3 (from mean of 9,450/ul to mean of 59,500/ul). These elevated counts persisted until G-CSF administration was discontinued five days later (See Figures 8 and 9).

Recombinant G-CSF having the amino acid sequence of canine G-CSF increased leukocyte counts (primarily neutrophils) and leukocyte counts were maintained at elevated levels as long as administration of the G-CSF was continued. Initial increases in leukocyte counts were most likely due to demargination of blood cells. The decrease in leukocyte counts observed following a reduced G-CSF dosage followed by a rapid return to elevated leukocyte levels with a full dosage demonstrate a rapid, dose-dependent response. There was no development of neutralizing antibodies to the G-CSF.

* * *

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

WHAT IS CLAIMED IS:

1. A purified and isolated polypeptide having part or all of the primary structural conformation and the biological properties of naturally-occurring canine granulocyte colony stimulating factor and characterized by being the product of prokaryotic or eukaryotic expression of an exogenous DNA sequence.

2. A polypeptide according to claim 1 wherein the exogenous DNA sequence is a genomic DNA sequence.

3. A polypeptide according to claim 1 wherein the exogenous DNA sequence is a cDNA sequence.

4. A polypeptide according to claim 1 wherein the exogenous DNA sequence is a manufactured DNA sequence.

5. A polypeptide according to claim 1 wherein the exogenous DNA sequence is carried on an autonomously replicating DNA plasmid or viral vector.

6. A polypeptide according to claim 1 possessing part or all of the primary structural conformation of canine granulocyte colony stimulating factor as set forth in Figure 2 or any naturally occurring allelic variant thereof.

7. A polypeptide according to claim 1 which has the immunological properties of naturally-occurring canine granulocyte colony stimulating factor.

8. A polypeptide according to claim 1 which has the <u>in vitro</u> biological activity of naturally-occurring canine granulocyte colony stimulating factor.

9. A DNA sequence for use in securing expression in a prokaryotic or eukaryotic host cell of a polypeptide product having at least a part of the primary structural conformation and the biological properties of naturally-occurring canine granulocyte colony stimulating factor, said DNA sequence set out in Figure 2 or its complimentary strand.

10. A prokaryotic or eukaryotic host cell transformed or transfected with a DNA sequence according to claim 9 in a manner allowing the host cell to express the polypeptide product.

11. A polypeptide product of the expression of a DNA sequence according to claim 9 in a prokaryotic or eukaryotic host cell.

12. A polypeptide product according to claim 11 wherein the polypeptide product is glycosylated or unglycosylated.

13. A purified and isolated DNA sequence coding for prokaryotic or eukaryotic host cell expression of a polypeptide having part or all of the primary structural conformation and the biological properties of canine granulocyte colony stimulating factor.

14. A genomic DNA sequence according to claim 13.

15. A cDNA sequence according to claim 13.

16. A DNA sequence according to claim 13 and including one or more codons preferred for expression in E. coli cells.

17. A DNA sequence according to claim 13 and including one or more codons preferred for expression in yeast cells.

18. A DNA sequence coding for a polypeptide fragment or polypeptide analog of naturally-occurring canine granulocyte colony stimulating factor.

19. A biologically functional plasmid or viral DNA vector containing a DNA sequence according to claim 9.

20. A prokaryotic or eukaryotic host cell stably transformed or transfected with the biologically functional plasmid or viral DNA vector according to claim 19.

21. A biologically functional plasmid or viral DNA vector containing a DNA sequence according to claim 13.

22. A prokaryotic or eukaryotic host cell stably transformed or transfected with the biologically functional plasmid or viral DNA vector according to claim 21.

23. A biologically functional plasmid or viral DNA vector containing a DNA sequence according to claim 18.

24. A prokaryotic or eukaryotic host cell stably transformed or transfected with the biologically functional plasmid or viral DNA vector according to claim 23.

25. A polypeptide product of the expression in a prokaryotic or eukaryotic host cell of a DNA sequence according to claims 13 or 18.

26. A synthetic polypeptide having part or all of the amino acid sequence as set forth in Figure 2 and having the in vitro biological activities of naturally-occurring canine granulocyte colony stimulating factor.

27. A synthetic polypeptide having part or all of the secondary conformation and part or all of the amino acid sequence set forth in Figure 2 and having the biological properties of naturally-occurring canine granulocyte colony stimulating factor.

28. A process for the production of a polypeptide having part or all of the primary structural conformation and the biological properties of naturally-occurring canine granulocyte colony stimulating factor, the process comprising: growing, under suitable nutrient conditions, prokaryotic or eukaryotic host cells transformed or transfected with a biologically functional plasmid or viral DNA vector according to claims 19, 21, or 23, and isolating desired polypeptide products of the expression of DNA sequences in the biologically functional plasmid or viral DNA vector.

29. A method for treating or preventing infections in a canine or feline animal comprising administering a composition which comprises a therapeutically effective treating amount or preventive amount of granulocyte colony stimulating factor.

30. A method according to claim 29 wherein said administering step comprises administering said granulocyte colony stimulating factor and one or more compounds selected from the group consisting of: GM-CSF, M-CSF, IL-3, interferon, IL-2, IL-6, TNF and a traditional antibiotic.

31. A method according to claim 29 wherein the composition is administered by the parenteral route.

32. A method according to claim 29 wherein the animal is a dog or cat.

33. A method according to claim 29 wherein the composition is a granulocyte colony stimulating factor having the human amino acid sequence.

34. A method according to claim 33 wherein the granulocyte colony stimulating factor is naturally-derived or is derived from genetically engineered host cells containing a genomic DNA sequence, a cDNA sequence or a manufactured DNA sequence encoding human granulocyte colony stimulating factor.

35. A method according to claim 29 wherein the composition is a granulocyte colony stimulating factor having the canine amino acid sequence.

36. A method according to claim 35 wherein the granulocyte colony stimulating factor is naturally-derived or is derived from genetically engineered host cells containing a genomic DNA sequence, a cDNA sequence or a manufactured DNA sequence encoding canine granulocyte colony stimulating factor.

37. A method according to claim 35 wherein the granulocyte colony stimulating factor is the polypeptide product of the expression in a prokaryotic or eukaryotic host cell of DNA sequence according to claims 9, 13, or 18.

38. A method for treating cancer in canine or feline animals comprising administering a composition which comprises a therapeutically effective treating or preventing amount of granulocyte colony stimulating factor in conjunction with chemotherapy.

39. A pharmaceutical composition for treating infections in canine or feline animals comprising a therapeutically effective amount of a granulocyte colony stimulating factor, in a pharmaceutically acceptable carrier.

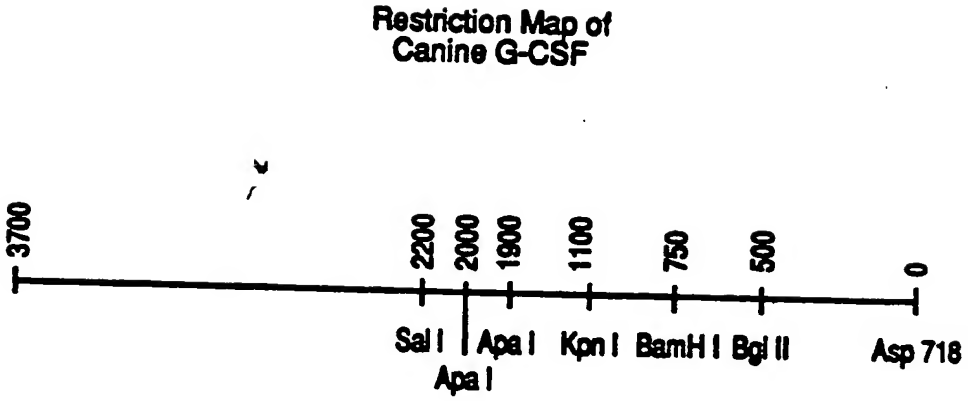

Restriction Map of
Canine G-CSF
3700
2200
2000
1900
1100
750
500
0
Sal I
Apa I
Apa I
Kpn I
BamH I
Bgl II
Asp 718

FIG. 1

```
Ala Pro Leu Gly Pro Thr Gly Pro Leu Pro Gln Ser Phe Leu Leu
gcc ccc ctg ggc cct acc ggc ccc ctg ccc cag agc ttc ctg ctc

Lys Cys Leu Glu Gln Met Arg Lys Val Gln Ala Asp Gly Thr Ala
aag tgc cta gag caa atg agg aag gtc cag gct gat ggc acg gcg

Leu Gln Glu Thr Leu Cys Ala Thr His Gln Leu Cys His Pro Glu
ctg cag gag acg ctg tgt gcc acc cac cag ctg tgc cat cct gag

Glu Leu Val Leu Leu Gly His Ala Leu Gly Ile Pro Gln Pro Pro
gag ttg gtg ctg ctc ggg cac gct ctg ggc atc ccc cag cct ccc

Leu Ser Ser Cys Ser Ser Gln Ala Leu Gln Leu Met Gly Cys Leu
ctg agc agc tgc tcc agc cag gcc ctg cag ctg atg ggc tgc ctg

Arg Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln
cgt caa ctc cac agc ggc ctc ttc ctc tac cag ggc ctc ctg cag
```

FIG. 2-A

```
Ala Leu Ala Gly Ile Ser Pro Glu Leu Ala Pro Thr Leu Asp Thr
gcc ctg gca ggg ata tcc ccc gag tta gcg ccc acc ttg gac aca

Leu Gln Leu Asp Thr Thr Asp Phe Ala Ile Asn Ile Trp Gln Gln
ctg cag ctg gac acc acc gac ttt gcc atc aac atc tgg cag cag

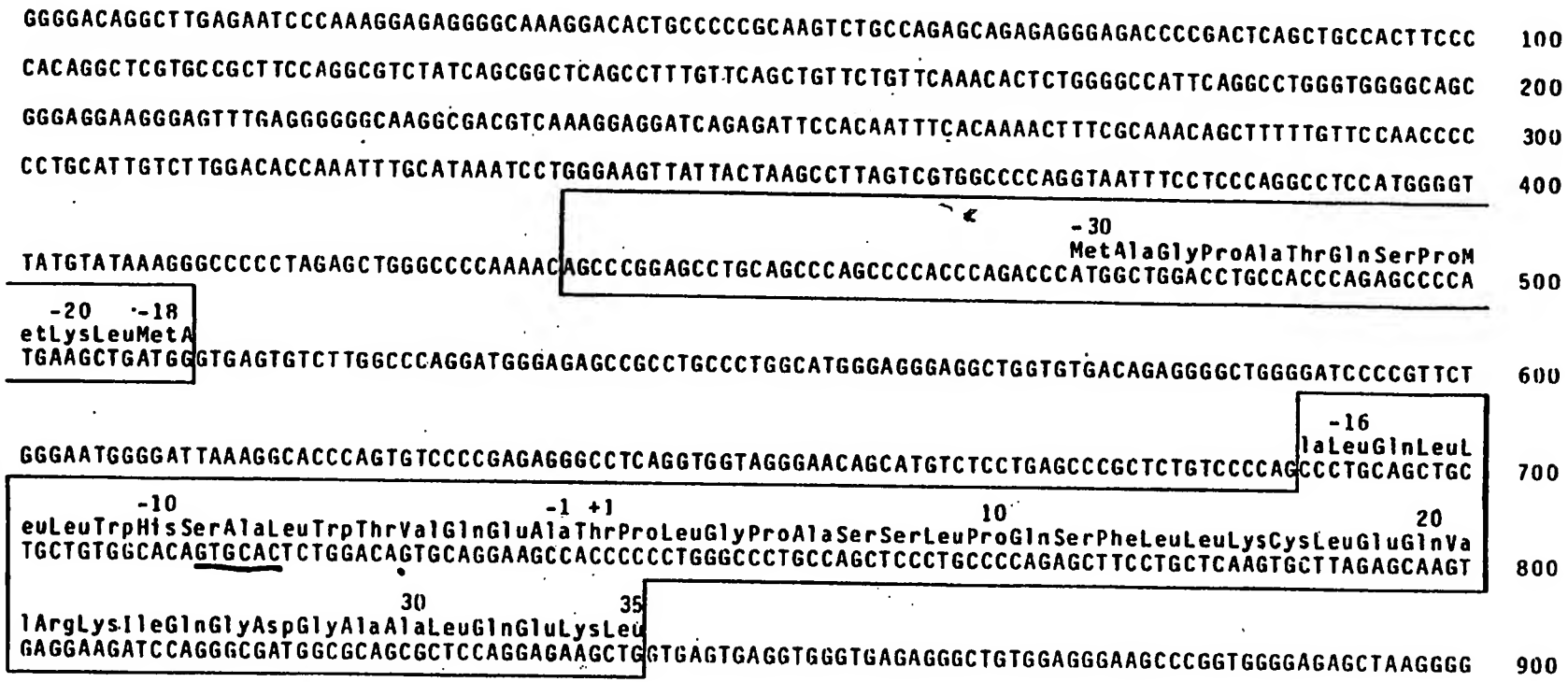
Met Glu Asp Leu Gly Met Ala Pro Ala Val Pro Pro Thr Gln Gly
atg gaa gat cta gga atg gcc ccc gcc gtg cca cct acc cag ggc

Thr Met Pro Ala Phe Thr Ser Ala Phe Gln Arg Arg Ala Gly Gly
acc atg cca gcc ttc acc tcg gcc ttc cag cgc cgg gca gga ggt

Val Leu Val Ala Ser Asn Leu Gln Ser Phe Leu Glu Leu Ala Tyr
gtc ctg gtg gcc tcc aac ctg cag agc ttc ctg gag ctg gca tat

Arg Ala Leu Arg His Phe Ala Lys Pro
cgc gct ctg cgc cac ttt gcc aaa ccc
```

FIG. 2-B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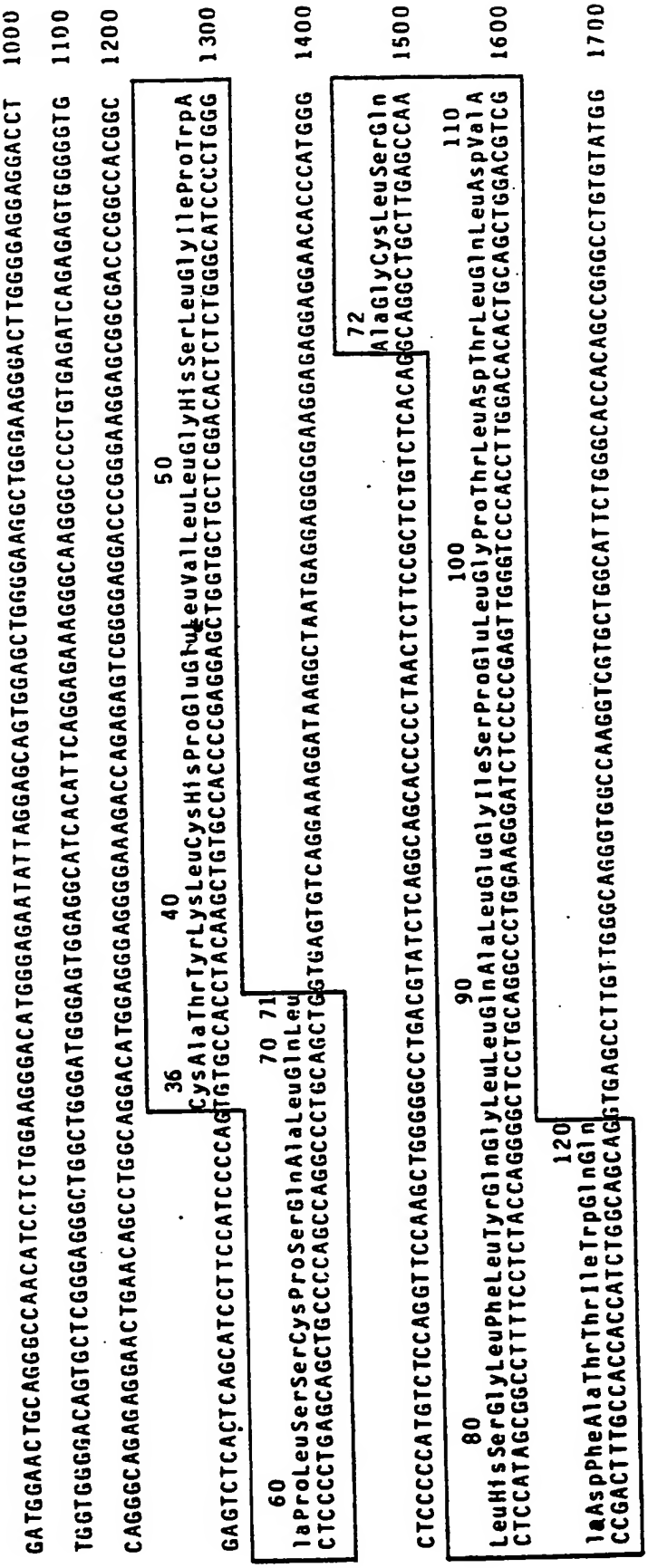
CACAGGCTCGTGCCGCTTCCAGGCGTCTATCAGCGGCTCAGCCTTTGTTCAGCTGTTCTGTTCAAACACTCTGGGGCCATTCAGGCCTGGGTGGGGCAGC 200
GGGAGGAAGGGAGTTTGAGGGGGGCAAGGCGACGTCAAAGGAGGATCAGAGATTCCACAATTTCACAAAACTTTCGCAAACAGCTTTTTGTTCCAACCCC 300
CCTGCATTGTCTTGGACACCAAATTTGCATAAATCCTGGGAAGTTATTACTAAGCCTTAGTCGTGGCCCCAGGTAATTTCCTCCCAGGCCTCCATGGGGT 400
-30
MetAlaGlyProAlaThrGlnSerProM
TATGTATAAAGGGCCCCCTAGAGCTGGGCCCCAAAACAGCCCGGAGCCTGCAGCCCAGCCCCACCCAGACCCATGGCTGGACCTGCCACCCAGAGCCCCA 500
-20 -18
etLysLeuMetA
TGAAGCTGATGGGTGAGTGTCTTGGCCCAGGATGGGAGAGCCGCCTGCCCTGGCATGGGAGGGAGGCTGGTGTGACAGAGGGGCTGGGGATCCCCGTTCT 600
-16
laLeuGlnLeuL
GGGAATGGGGATTAAAGGCACCCAGTGTCCCCGAGAGGGCCTCAGGTGGTAGGGAACAGCATGTCTCCTGAGCCCGCTCTGTCCCCAGCCCTGCAGCTGC 700
-10 -1 +1 10 20
euLeuTrpHisSerAlaLeuTrpThrValGlnGluAlaThrProLeuGlyProAlaSerSerLeuProGlnSerPheLeuLeuLysCysLeuGluGlnVa
TGCTGTGGCACAGTGCACTCTGGACAGTGCAGGAAGCCACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCAAGTGCTTAGAGCAAGT 800
30 35
lArgLysIleGlnGlyAspGlyAlaAlaLeuGlnGluLysLeu
GAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAGGAGAAGCTGGTGAGTGAGGTGGGTGAGAGGGCTGTGGAGGGAAGCCCGGTGGGGAGAGCTAAGGGG 900

FIG. 3-A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CAGGGCAGAGAGGAACTGAACAGCCTGGCAGGACATGGAGGGAGGGGAAAGACCAGAGAGTCGGGGAGGACCCGGGAAGGAGCGGCGACCCGGCCACGGC 1200
GAGTCTCACTCAGCATCCTTCCATCCCCAG 1300
1aProLeuSerSerCysProSerGlnAlaLeuGlnLeu 1400
CTCCCCTGAGGCAGCTGCCCCAGCCAGGCCCTGCAGCTG 1500
CTCCCCCATGTCTCCAGGTTCCAAGCTGGGGGCCTGACGTATCTCAGGCAGCACCCCCTAACTCTTCCGCTCTGTCTCACAG 1600
LeuHisSerGlyLeuPheLeuTyrGlnGlyLeuLeuGlnAlaLeuGluGlyIleSerProGluLeuGlyProThrLeuAspThrLeuGlnLeuAspValA
CTCCATAGCGGCCTTTTCCTCTACCAGGGGCTCCTGCAGGCCCTGGAAGGGATCTCCCCCGAGTTGGGTCCCACCTTGGACACACTGCAGCTGGACGTCG 1600
1aAspPheAlaThrThrIleTrpGlnGln 120
CCGACTTTGCCACCACCATCTGGCAGCAG 1700

FIG. 3-B

```
                                                                                         121
                                                                                         MetGluG
GCCCTGTCCATGCTGTCAGCCCCCAGCATTTCCTCATTTGTAATAACGCCCACTCAGAAGGGCCCAACCACTGATCACAGCTTTCCCCCACAGATGGAAG     1800

                   130                          140                               150
luLeuGlyMetAlaProAlaLeuGlnProThrGlnGlyAlaMetProAlaPheAlaSerAlaPheGlnArgArgAlaGlyGlyValLeuValAlaSerHi
AACTGGGAATGGCCCCTGCCCTGCAGCCCACCCAGGGTGCCATGCCGGCCTTCGCCTCTGCTTTCCAGCGCCGGGCAGGAGGGGTCCTGGTTGCCTCCCA     1900

       160                           170          174
sLeuGlnSerPheLeuGluValSerTyrArgValLeuArgHisLeuAlaGlnProOP
TCTGCAGAGCTTCCTGGAGGTGTCGTACCGCGTTCTACGCCACCTTGCCCAGCCCTGAGCCAAGCCCTCCCCATCCCATGTATTTATCTCTATTTAATAT     2000

TTATGTCTATTTAAGCCTCATATTTAAAGACAGGGAAGAGCAGAACGGAGCCCCAGGCCTCTGTGTCCTTCCCTGCATTTCTGAGTTTCATTCTCCTGCC     2100

TGTAGCAGTGAGAAAAAGCTCCTGTCCTCCCATCCCCTGGACTGGGAGGTAGATAGGTAAATACCAAGTATTTATTACTATGACTGCTCCCCAGCCCTGG     2200

CTCTGCAATGGGCACTGGGATGAGCCGCTGTGAGCCCCTGGTCCTGAGGGTCCCCACCTGGGACCCTTGAGAGTATCAGGTCTCCCACGTGGGAGACAAG     2300

AAATCCCTGTTTAATATTTAAACAGCAGTGTTCCCCATCTGGGTCCTTGCACCCCTCACTCTGGCCTCAGCCGACTGCACAGCGGCCCCTGCATCCCCTT     2400

GGCTGTGAGGCCCCTGGACAAGCAGAGGTGGCCAGAGCTGGGAGGCATGGCCCTGGGGTCCCACGAATTTGCTGGGGAATCTCGTTTTTCTTCTTAAGAC     2500

TTTTGGGACATGGTTTGACTCCCGAACATCACCGACGTGTCTCCTGTTTTTCTGGGTGGCCTCGGGACACCTGCCCTGCCCCCACGAGGGTCAGGACTGT     2600

GACTCTTTTTAGGGCCAGGCAGGTGCCTGGACATTTGCCTTGCTGGATGGGGACTGGGGATGTGGGAGGGAGCAGACAGGAGGAATCATGTCAGGCCTGT     2700

GTGTGAAAGGAAGCTCCACTGTCACCCTCCACCTCTTCACCCCCCACTCACCAGTGTCCCCTCCACTGTCACATTGTAACTGAACTTCAGGATAATAAAG     2800

TGTTTGCCTCCAGTCACGTCCTTCCTCCTTCTTGAGTCCAGCTGGTGCCTGGCCAGGGGCTGGGGAGGTGGCTGAAGGGTGGAGAGGCCAGAGGGAGGT     2900

CGGGGAGGAGGTCTGGGGAGGAGGTCCAGGGAGGAGGAGGAAAGTTCTCAAGTTCGTCTGACATTCATTCCGTTAGCACATATTTATCTGAGCACCTACT     3000

CTGTGCAGACGCTGGGCTAAGTGCTGGGGACACAGCAGGGAACAAGGCAGACATGGAATCTGCACTCGAG                                  3070
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FIG. 3-C

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                                                                  60
  C TAG AAA AAA CCA AGG AGG TAA TAA ATA ATG GCA CCT TTA GGT CCA ACT GGT CCT CTG
                                    Met Ala Pro Leu Gly Pro Thr Gly Pro Leu
                30                                                        120
CCT CAA AGT TTC CTG CTG AAA TGC CTC GAG CAG ATG CGT AAA GTT CAA GCT GAT GGT ACC
Pr  Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Met Arg Lys Val Gln Ala Asp Gly Thr
                90                                                       180
GCA CTC CAA GAA ACT CTG TGC GCA ACT CAC CAA CTG TGC CAC CCT GAA GAA CTC GTA CTG
Ala Leu Gln Glu Thr Leu Cys Ala Thr His Gln Leu Cys His Pro Glu Glu Leu Val Leu
               150                                                       240
CTC GGT CAC GCA CTC GGT ATT CCG CAG CCG CCG CTG TCT TCT TGC TCC TCT CAG GCT CTG
Leu Gly His Ala Leu Gly Ile Pro Gln Pro Pro Leu Ser Ser Cys Ser Ser Gln Ala Leu
               210                                                       300
CAA CTC ATG GGT TGC CTC CGT CAA CTG CAT TCT GGC CTG TTC CTG TAC CAG GGT CTC CTG
Gln Leu M t Gly Cys Leu Arg Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu
               270
```

FIG. 4-A

```
                                                                  360
CAA GCT TTG GCT GGC ATC TCT CCG GAA CTC GCA CCT ACT CTC GAC ACT CTG CAG CTC GAC
Gln Ala Leu Ala Gly Ile Ser Pro Glu Leu Ala Pro Thr Leu Asp Thr Leu Gln Leu Asp

          330                                                     420
ACT ACC GAC TTC GCT ATC AAC ATT TGG CAG CAA ATG GAA GAT CTG GGC ATG GCA CCG GCT
Thr Thr Asp Phe Ala Ile Asn Ile Trp Gln Gln Met Glu Asp Leu Gly Met Ala Pro Ala

          390                                                     480
GTT CCG CCG ACT CAG GGC ACT ATG CCT GCT TTT ACT TCT GCT TTC CAG CGT CGT GCT GGT
Val Pro Pro Thr Gln Gly Thr Met Pro Ala Phe Thr Ser Ala Phe Gln Arg Arg Ala Gly

          450                                                     540
GGT GTA CTC GTA GCT TCT AAC CTC CAG TCT TTC CTC GAA CTC GCT TAC CGT GCT CTG CGT
Gly Val Leu Val Ala Ser Asn Leu Gln Ser Phe Leu Glu Leu Ala Tyr Arg Ala Leu Arg

          510
CAC TTC GCT AAA CCG TAA TAG GAT C
His Phe Ala Lys Pro End End Asp
```

FIG. 4-B

```
                10                  20                  30                  40                  50                  60
          CTAGAAAA AACCAAGGAG GTAATAAATA ATGGCACCTT TAGGTCCAAC TGGTCCTCTG
          xbaI TTTT TTGGTTCCTC CATTATTTAT TACCGTGGAA ATCCAGGTTG ACCAGGAGAC

                70                  80                  90                 100                 110                 120
CCTCAAAGTT TCCTGCTCAA ATGCCTCGAG CAGATGCGTA AAGTTCAAGC TGATGGTACC
GGAGTTTCAA AGGACGACTT TACGGAGCTC GTCTACGCAT TTCAAGTTCG ACTACCATGG

               130                 140                 150                 160                 170                 180
GCACTCCAAG AAACTCTGTG CGCAACTCAC CAACTGTGCC ACCCTGAAGA ACTCGTACTG
CGTGAGGTTC TTTGAGACAC GCGTTGAGTG GTTGACACGG TGGGACTTCT TGAGCATGAC

               190                 200                 210                 220                 230                 240
CTCGGTCACG CACTCGGTAT TCCGCAGCCG CCGCTGTCTT CTTGCTCCTC TCAGGCTCTG
GAGCCAGTGC GTGAGCCATA AGGCGTCGGC GGCGACAGAA GAACGAGGAG AGTCCGAGAC

               250                 260                 270                 280                 290                 300
CAACTCATGG GTTGCCTCCG TCAACTGCAT TCTGGCCTGT TCCTGTACCA GGGTCTCCTG
GTTGAGTACC CAACGGAGGC AGTTGACGTA AGACCGGACA AGGACATGGT CCCAGAGGAC
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FIG. 5-A

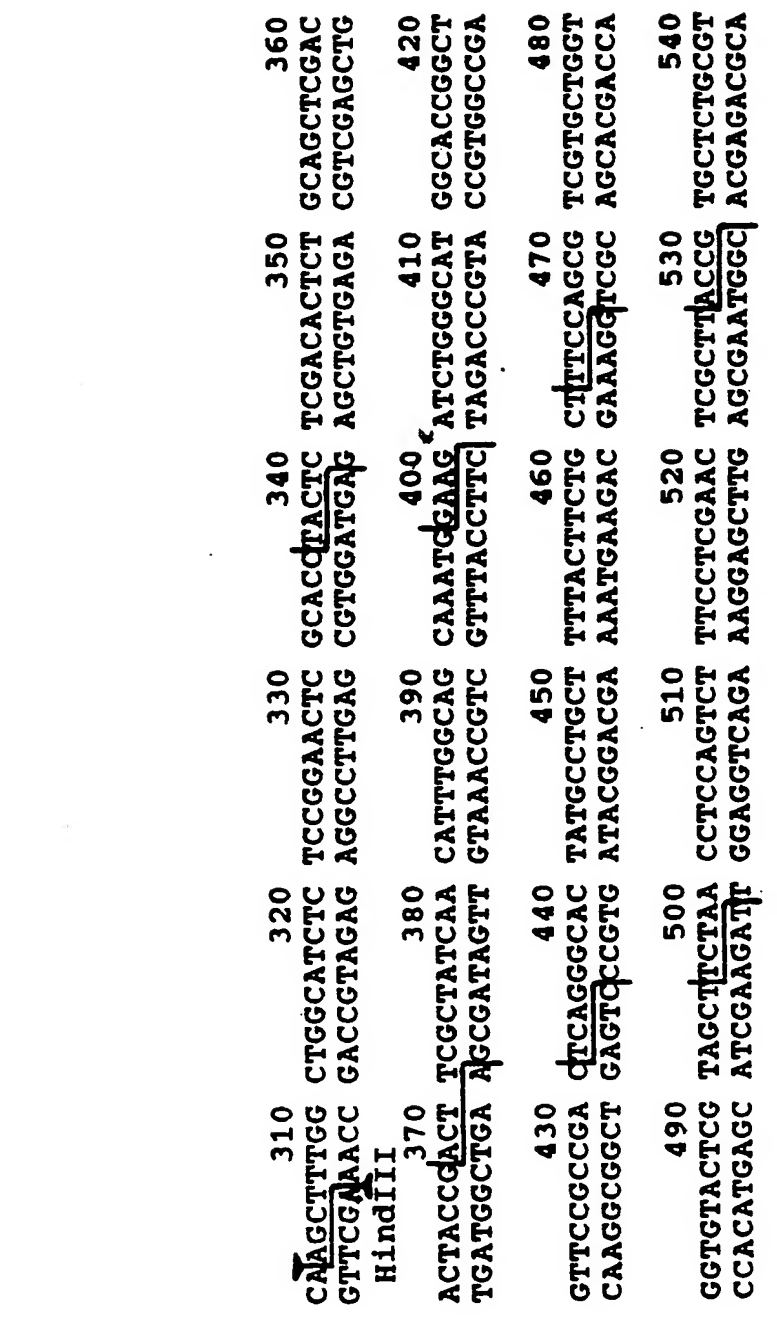
310 320 330 340 350 360
CAAGCTTTGG CTGGCATCTC TCCGGAACTC GCACCTACTC TCGACACTCT GCAGCTCGAC
GTTCGAAACC GACCGTAGAG AGGCCTTGAG CGTGGATGAG AGCTGTGAGA CGTCGAGCTG
HindIII
370 380 390 400 410 420
ACTACCGACT TCGCTATCAA CATTTGGCAG CAAATGGAAG ATCTGGGCAT GGCACCGGCT
TGATGGCTGA AGCGATAGTT GTAAACCGTC GTTTACCTTC TAGACCCGTA CCGTGGCCGA
430 440 450 460 470 480
GTTCCGCCGA CTCAGGGCAC TATGCCTGCT TTTACTTCTG CTTTCCAGCG TCGTGCTGGT
CAAGGCGGCT GAGTCCCGTG ATACGGACGA AAATGAAGAC GAAAGGTCGC AGCACGACCA
490 500 510 520 530 540
GGTGTACTCG TAGCTTCTAA CCTCCAGTCT TTCCTCGAAC TCGCTTACCG TGCTCTGCGT
CCACATGAGC ATCGAAGATT GGAGGTCAGA AAGGAGCTTG AGCGAATGGC ACGAGACGCA
550 560
CACTTCGCTA AACCGTAATA G BamH1
GTGAAGCGAT TTGGCATTAT CCTAG

FIG. 5-B

```
                10                  20                  30                  40                  50                  60
CTAGAAAAAA CCAAGGAGGT AATAAATAAT GGCACCTTTA GGTCCAACTG GTCCTCTGCC
TTTTTT GGTTCCTCCA TTATTTATTA CCGTGGAAAT CCAGGTTGAC CAGGAGACGG
xbaI

                70                  80                  90                  100                 110                 120
TCAAAGTTTC CTGCTCGAAAT GCCTCGAGCA GATGCGTAAA GTTCAAGCTG ATGGTACCGC
AGTTTCAAAG GACGACTTTA CGGAGCTCGT CTACGCATTT CAAGTTCGAC TACCATGGCG

                130                 140                 150                 160                 170                 180
ACTCCAAGAA ACTCTGTGCG CAACTCAGCA ACTGTGCCAC CCTGAAGAAC TCGTACTGCT
TGAGGTTCTT TGAGACACGC GTTGAGTCGT TGACACGGTG GGACTTCTTG AGCATGACGA

                190                 200                 210                 220                 230                 240
CGGTCACGCA CTCGGTATTC CGCAGCCGCC GCTGTCTTCT TGCTCCTCTC AGGCTCTGCA
GCCAGTGCGT GAGCCATAAG GCGTCGGCGG CGACAGAAGA ACGAGGAGAG TCCGAGACGT

                250                 260                 270                 280                 290                 300
ACTCATGGGT TGCCTCCGTC AACTGCATTC TGGCCTGTTC CTGTACCAGG GTCTCCTGCA HindIII
TGAGTACCCA ACGGAGGCAG TTGACGTAAG ACCGGACAAG GACATGGTCC CAGAGGACGT TCGA
```

FIG. 6-A

```
         10         20         30         40         50         60
AGCTTTGGCT GGCATCTCTC CGGAACTCGC ACCTACTCTC GACACTCTGC AGCTCGACAC
    AACCGA CCGTAGAGAG GCCTTGAGCG TGGATGAGAG CTGTGAGACG TCGAGCTGTG
HindIII
         70         80         90        100        110        120
TACCGACTTC GCTATCAACA TTTGGCAGCA AATGGAAGAT CTGGGCATGG CACCGGCTGT
ATGGCTGAAG CGATAGTTGT AAACCGTCGT TTACCTTCTA GACCCGTACC GTGGCCGACA

        130        140        150        160        170        180
TCCGCCGACT CAGGGCACTA TGCCTGCTTT TACTTCTGCT TTCCAGCGTC GTGCTGGTGG
AGGCGGCTGA GTCCCGTGAT ACGGACGAAA ATGAAGACGA AAGGTCGCAG CACGACCACC

        190        200        210        220        230        240
TGTACTCGTA GCTTCTAACC TCCAGTCTTT CCTCGAACTC GCTTACCGTG CTCTGCGTCA
ACATGAGCAT CGAAGATTGG AGGTCAGAAA GGAGCTTGAG CGAATGGCAC GAGACGCAGT

        250        260
CTTCGCTAAA CCGTAATAG     BamH1
GAAGCGATTT GGCATTATCC TAG
```

FIG. 6-B

```
1   MetAlaProLeuGlyProThrGlyProLeuProGlnSerPheLeuLeuLysCysLeuGlu
    |  :  |  |  |  |  :        |  |  |  |  |  |  |  |  |  |  |
1   MetThrProLeuGlyProAlaSerSerLeuProGlnSerPheLeuLeuLysCysLeuGlu

21  GlnMetArgLysValGlnAlaAspGlyThrAlaLeuGlnGluThrLeuCysAlaThrHis
    |  :  |  |  :  |  |  |  :  |  |  |  |  |  |  |  |  |  |  |
21  GlnValArgLysIleGlnGlyAspGlyAlaAlaLeuGlnGluLysLeuCysAlaThrTyr

41  GlnLeuCysHisProGluGluLeuValLeuLeuGlyHisAlaLeuGlyIleProGlnPro
    |  |  |  |  |  |  |  |  |  |  :  |  |  |  |  |  |  |  |  |
41  LysLeuCysHisProGluGluLeuValLeuLeuGlyHisSerLeuGlyIleProTrpAla

61  ProLeuSerSerCysSerGlnAlaLeuGlnLeuMetGlyCysLeuArgGlnLeuHis
    |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
61  ProLeuSerSerCysProSerGlnAlaLeuGlnLeuAlaGlyCysLeuSerGlnLeuHis

81  SerGlyLeuPheLeuTyrGlnGlyLeuLeuGlnAlaLeuAlaGlyIleSerProGluLeu
    |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
81  SerGlyLeuPheLeuTyrGlnGlyLeuLeuGlnAlaLeuGluGlyIleSerProGluLeu
```

FIG. 7-A

```
101  AlaProThrLeuAspThrLeuGlnLeuAspThrThrAspPheAlaIleAsnIleTrpGln
         |  |  |  |  |  |  |  |  |     :  |  |  |        |  |  |
101  GlyProThrLeuAspThrLeuGlnLeuAspValAlaAspPheAlaThrThrIleTrpGln

121  GlnMetGluAspLeuGlyMetAlaProAlaValProProThrGlnGlyThrMetProAla
      |  |  |  :  |  |  |  |  |  |  :     |  |  |  |  :  |  |  |
121  GlnMetGluGluLeuGlyMetAlaProAlaLeuGlnProThrGlnGlyAlaMetProAla

141  PheThrSerAlaPheGlnArgArgAlaGlyGlyValLeuValAlaSerAsnLeuGlnSer
      |  :  |  |  |  |  |  |  |  |  |  |  |  |  |  |     |  |  |
141  PheAlaSerAlaPheGlnArgArgAlaGlyGlyValLeuValAlaSerHisLeuGlnSer

161  PheLeuGluLeuAlaTyrArgAlaLeuArgHisPheAlaLysPro
      |  |  |  :  :  |  |     |  |  |     |     |
161  PheLeuGluValSerTyrArgValLeuArgHisLeuAlaGlnPro
```

FIG. 7-B

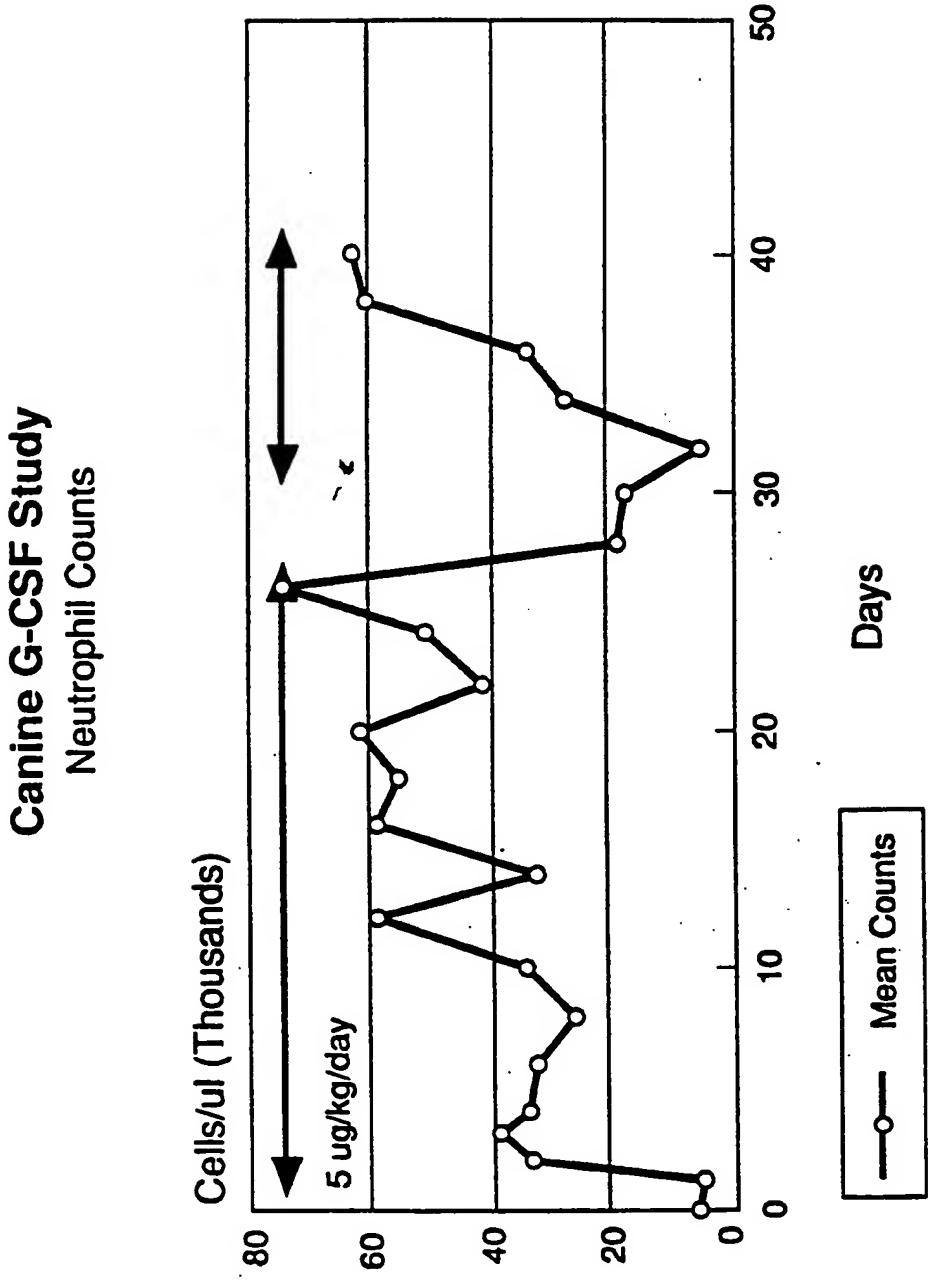
Canine G-CSF Study
Neutrophil Counts
Cells/ul (Thousands)
80
60
40
20
0
5 ug/kg/day
0
10
20
30
40
50
Days
Mean Counts

FIG. 8

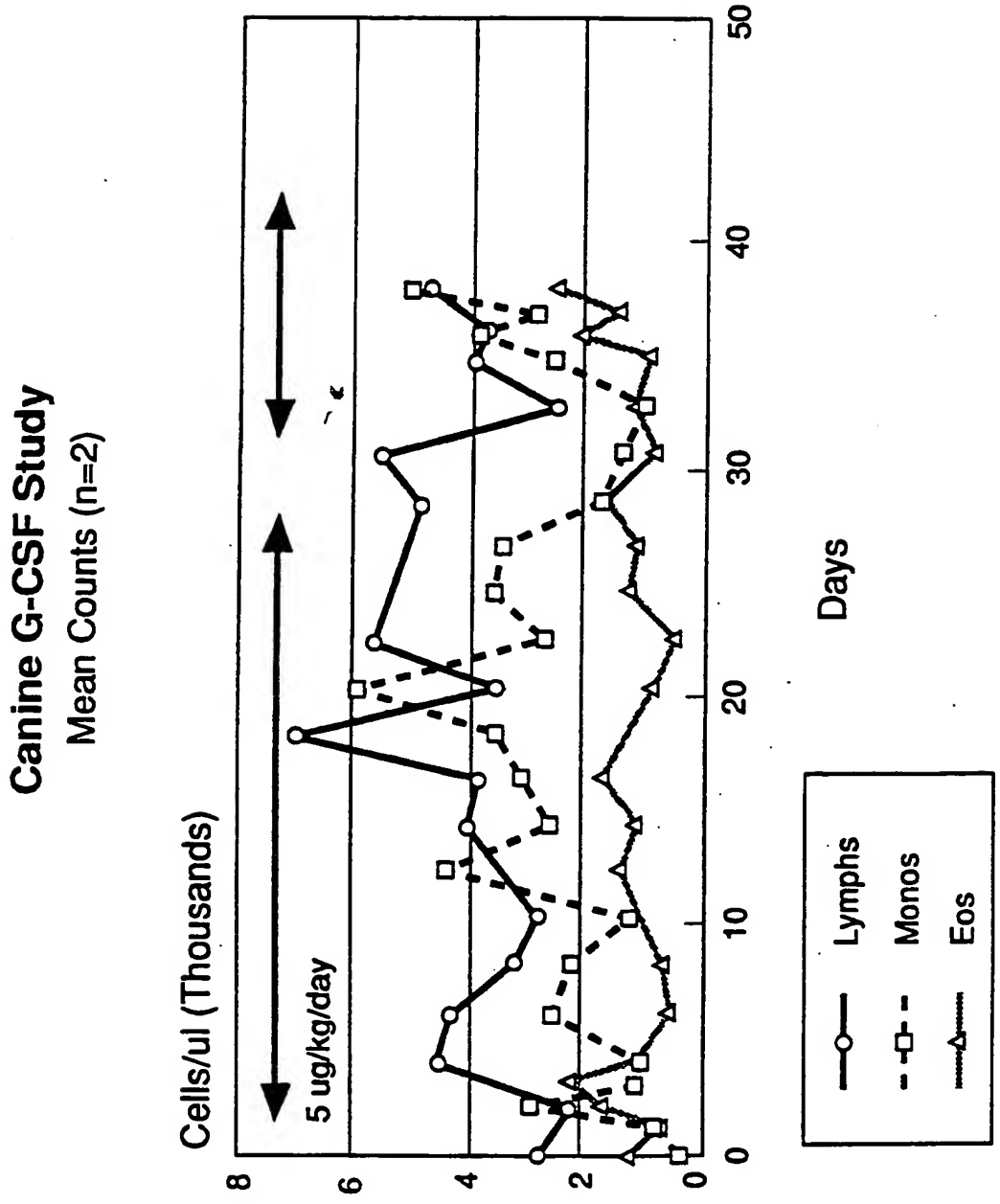
Canine G-CSF Study
Mean Counts (n=2)
Cells/ul (Thousands)
8
6
4
2
0
0
10
20
30
40
50
Days
5 ug/kg/day
Lymphs
Monos
Eos

FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/05522

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 13/00; A61K 37/02; C12P 21/02; C12N 15/24; C07H 15/12
U.S.CL.: 530/351; 435/69.5,172.3,240.2,243,320; 536/27; 424/85.1

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.CL.	530/351,395,300,820,827; 435/69.5,172.3,240.2,243,320; 424/85.1; 514/2,8,12; 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

COMPUTER DATA-BASE SEARCH ON CAS FOR: CANINE OR FELINE AND G-CSF, AND (DNA OR RECOMBINANT)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,833,127 (Ono et al.) 23 May 1989, see entire document.	1-39
Y	US, A, 4,810,643 (Souza) 07 March 1989, see entire document.	1-39
Y,P	US, A, 4,904,584 (Shaw) 27 February 1990 see entire document.	1-39
Y	EP, A, 0,220,520 (Yamozaki) 05 June 1987, see the entire document.	1-39
Y	Proc. Natl. Acad. Sci., Vol. 83, Issued October 1986, Tsuchiya et al., "Isolation and Characterization of the cDNA for Murine Granulocyte Colony-Stimulating Factor", pages 7633-37, (see pages 7633 and 7635-30).	1-39

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [1]	Date of Mailing of this International Search Report [2]
13 December 1990	04 FEB 1991
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	GARNETTE D. DRAPER, PRIMARY EXAMINER

FURTHER INF RMATION CONTINUED FROM THE SECOND SHEET

Y	The EMBO Journal, Vol. 6, No. 3, Issued 1987, (Tsuchiya et al.), "Characterization of Recombinant Human Granulocyte-Colony-Stimulating Factor Produced in Mouse Cells", pages 611-616, (see pages 611 and 614-15).	1-39

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers .... because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows: Group I, Claims 1-8,11-12,25-27,39 to polypeptide to G-CSF and composition; classified in 530/351. Group II, claims 9-10,13-24 and 28 to DNA, cell lines plasmid and recombinant production of protein, classified in 435/69.5,172.3. Group III, claims 29-38 to a method of treatment, classified in 424/85.1.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☒ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.